## ACE2 Activation for Treatment of Heart, Lung and Kidney Disease and Hypertension

### FIELD OF THE INVENTION

5 The present invention provides compositions and methods for use in diagnosing and treating heart, lung and kidney diseases, including hypertension, coronary heart disease, heart and kidney failure, lung edema, and lung injury such as in toxic shock or artificial ventilation.

### BACKGROUND OF THE INVENTION

Cardiovascular disease will be the number one health care burden of the 21$^{st}$
10 century, and is predicted to be the most common cause of death worldwide by 2020. A major risk factor for heart disease is high blood pressure. Hypertension is a multifactorial quantitative trait controlled by both genetic and environmental factors. While much is known about environmental factors that can contribute to high blood pressure, such as diet and physical activity, less
15 is known about the genetic factors that are responsible for predisposition to cardiovascular disease. Despite the identification of several putative genetic quantitative trait loci (QTL) associated with hypertension in animal models, none of these loci have been translated into genes. Thus, the molecular and genetic mechanisms underlying hypertension and other cardiovascular
20 diseases remain largely obscure.

One critical regulator of blood pressure homeostasis is the renin-angiotensin system (RAS). The protease renin cleaves angiotensinogen into the inactive decameric peptide angiotensin I (AngI). The action of angiotensin-converting enzyme (ACE) then catalyzes the cleavage of the AngI into the active octomer
25 angiotensin II (AngII), which can contribute to hypertension by promoting vascular smooth muscle vasoconstriction and renal tubule sodium reabsorption. ACE mutant mice display spontaneous hypotension, partial male infertility, and kidney malformations. In humans, an ACE polymorphism has been associated with determinants of renal and cardiovascular function,
30 and pharmacological inhibition of ACE and AngII receptors are effective in

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lowering blood pressure and kidney disease. In addition, inhibition of ACE and AngII receptors has beneficial effects in heart failure.

Recently a homologue of ACE, termed ACE2, has been identified which is predominantly expressed in the vascular endothelial cells of the kidney and
5    heart. Interestingly, two ACE homologues also exist in flies. Unlike ACE, ACE2 functions as a carboxypeptidase, cleaving a single residue from AngI, generating Ang1-9, and a single residue form AngII to generate Ang1-7. These *in vitro* biochemical data suggested that, ACE2 modulates the RAS and thus may play a role in blood pressure regulation. The *in vivo* role of
10   ACE2 in the cardiovascular system and the RAS is not known.        ,

Acton *et al.* in U.S. Patent 6,194,556, describe the use of ACE2 in diagnosis and therapeutics of ACE2 associated states. The patent stated that ACE2 expression levels increase with hypertension and that antagonists or inhibitors of ACE2 activity would be useful in the treatment of increased blood pressure
15   or related disorders. Canadian patent application no. 2,372,387 provides specific examples of ACE2 inhibitors which, are intended to be useful for the treatment of heart disease, such as hypertension. This again emphasizes the need to inhibit, rather than increase, ACE2 activity. These references, which teachthe need to inhibit ACE2 activity, are based only on *in vitro* experimental
20   data. They do not provide *in vivo* data, such as knock out mammal data, to characterize ACE2. To date, no ACE2 inhibitors have been approved as pharmaceuticals for treatment of hypertension. Furthermore, the *in vivo* role of ACE2 in the cardiovascular system and the RAS remains largely unknown. There remains a need to characterize the function of ACE2 in order to be able
25   to design appropriate diagnostic tests and pharmaceuticals for treatment of heart and kidney disease.

## SUMMARY OF THE INVENTION

The invention provides a new paradigm for the regulation of the renin-angiotensin system and shows a completely new and unexpected usage of
30   ACE2, in contrast to prediction based on *in vitro* data (Acton patent) and

unexpected in the previous art, as a critical negative regulator of the RAS required for heart function and blood pressure control. Activation of ACE2 is critical for treatment and prevention of heart, lung and kidney disease. The invention shows for the first time that administering an ACE2 activator to an

5    animal prevents and treats hypertension and cardiac and kidney disease, and lung injury.

## BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings in which:

10    Figure 1. Sequence and chromosomal mapping of rat *ACE2*.

*a,* Protein alignment of rat, mouse and human ACE2, with mouse and human testis-ACE (T-ACE). Blackened shading indicates amino acid identity and gray shading indicates degree of amino acid similarity. *b,* Schematic domain structure of ACE and ACE2. Note that ACE2 only contains one ACE-domain

15    with the consensus zinc binding site HEMGH. The catalytic centers are indicated in black, the signal peptide is indicated in grey and the transmembrane domain in hatched lines. *c,* Expression patterns of mouse and rat ACE2 genes in different adult tissues and different days of embryonic development (E7 = embryonic day 7). Note that two isoforms are present for

20    ACE2 in mice, but not in rat or human (not shown), a feature similar to that seen for ACE[15]. *d,* Results of radiation hybrid mapping of rat *ACE2,* compared to the mapping of a QTL identified in Sabra salt-sensitive animals (*SS-X*), SHRSP (*BP3*), and SHR rats (BB.Xs). Polymorphic marker names are indicated to the left of the ideogram. LOD scores and theta values for markers

25    linked to *ACE2* are shown. cR = centiRads.

Figure 2. Expression levels of ACE2 in rat models of hypertension.

*a,* Northern blot analysis of *ACE2* mRNA from kidneys of Sabra SBH/y and SBN/y rats. Upper section shows representation of Northern blots with actin control levels. Lower panel shows relative levels of *ace2* message normalized

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to actin levels. *b*, Western blot analysis of ACE2 protein levels from kidneys of Sabra SBH/y and their control SBN/y rats, as well as SHR and SHRSP and their control WKY rats. Upper section shows representative Western blots. Systolic blood pressure (BP) in mmHg for the respective Sabra rats is indicated. Lower panel shows relative protein levels of ACE2 corrected for actin. Bars show mean values +/- SEM. * = $p < 0.05$, ** = $p < 0.01$. (n=4, for all groups).

Figure 3. Targeted disruption of mouse ACE2 by homologous recombination.

*a*, Gene targeting strategy. A portion of the murine *ace2* wild-type locus (top) is shown. Black boxes indicate exons. The targeting vector was designed to replace exon 9 encoding the zinc binding catalytic domain with the neomycin (neo) resistance gene cassette placed in the anti-sense orientation. Thymidine kinase (TK) was used for negative selection. The 3' and 5' flanking probes used for Southern analysis are indicated with a hatched box. *b*, Southern blot analysis of $ace^{+/y}$ and $ace^{-/y}$ ES cells. Genomic DNA was digested with EcoRI and hybridized to the 3' and 5' flanking probe shown in (a). *c*, Western blot analysis of ACE2 protein expression in the kidneys of $ace2^{+/y}$ $ace^{-/y}$ mice. The anti-ACE2 Ab is reactive to a region N-terminal to the deletion. *d*, RT-PCR analysis of ACE mRNA expression in the heart and kidneys of $ace2^{+/y}$ and $ace2^{-/y}$ mice. Different PCR cycles for linear amplification and GAPDH mRNA levels as a control are shown.

Figure 4. Normal blood pressure and kidney functions

*a*, Blood pressure measurements in 3 month old $ace2^{+/y}$ (n= 8) and $ace2^{-/y}$ (n = 8) mice in the absence (left panels) or presence of the ACE blocker captopril. Blood pressures were determined by using tail cuffing and mean values +/- SD are shown. Captopril was administered to mice for 2 weeks prior to blood pressure measurements as described in Methods. These blood pressures were confirmed using invasive hemodynamic and Langendorf measurements (not shown). The differences in both captopril-treated $ace2^{+/y}$ and $ace2^{-/y}$ mice are significantly different to that of their respective untreated

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groups (** p< 0.01). *b,* Normal kidney histologies were seen in 6 month old *ace2$^{+/y}$* and *ace2$^{-/y}$* mice. Arrows indicate glomeruli. Figure 5. Heart morphology

5    *a,* H&E stained sections of hearts isolated from 6 month old *ace2$^{+/y}$* and *ace2$^{-}$$^{/y}$* mice. Enlarged left ventricles (LV) and right ventricles (RV) was observed in *ace2$^{-/y}$* mice. However, the overall heart size was comparable between both genotypes and there was no evidence of cardiac hypertrophy, macroscopically or in isolated cardiomyocytes. *b,* Quantitation of heart/body weight ratios from 6 month old *ace2$^{+/y}$* (n= 8) and *ace2$^{-/y}$*(n=8) mice as an

10   indicator of cardiac hypertrophy. It should be noted that the body weights, heart weights, tibial lengths, and the heart weight/tibial length ratios were also not changed between the different genetic groups at all ages analyzed (not shown). *c,d,* There was an absence of interstitial fibrosis in *ace2$^{-/y}$* mice. One hallmark feature for dilated cardiomyopathy is interstitial fibrosis. However,

15   interstitial fibrosis was comparable between the hearts of *ace2$^{+/y}$* (n= 8) and *ace2$^{-/y}$*(n=8) mice. (c) shows PSR staining of individual hearts. Note the normal perivascular fibrosis, stained in red, in both wild type and mutant animals. (d) quantitation of fibrotic changes in the interstitium.

Figure 6. Loss of ACE2 results in severe contractile heart failure

20   *a,* Echocardiographic measurements of contracting hearts in a 6 months old *ace2$^{+/y}$* and two *ace2$^{-/y}$* mice. Peaks and valleys indicate the systole and diastole of individual heart beats. Arrows indicate the distance between systolic contraction (LVESD) and diastolic relaxation (LVEDD), values that determine the percentage of fractional shortening (% FS). Note the increased

25   diastolic and systolic dimensions in the *ace2$^{-/y}$* mice indicative of cardiac dilation. The experimental data can be seen in Table 1. *b,* Percentage fractional shorting and velocity of circumferential fiber shortening, two hallmark parameters for heart contraction was seen, in 6 month old *ace2$^{+/y}$* (n=8) and *ace2$^{-/y}$* (n=8) mice and 6 month old *ace2$^{+/-}$* (n=5) and *ace2$^{-/-}$* (n=5)

30   female mice. Values were determined by echocardiography. Mean values +/- SD are shown. • p < 0.05 and •• p < 0.01 between genetic groups. The

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experimental data can be seen in Table 1.c, Blood pressure measurements in 6 month old male ace2$^{+/y}$ (n = 8) and ace2$^{-/y}$ (n = 8) and 6 months old female mice ace2$^{+/-}$ (n = 5) and ace2$^{-/-}$ (n = 5) female mice. Mean values +/- SD are shown. Blood pressures were confirmed using invasive hemodynamic measurements as can be seen in Table 2.. • p < 0.05.

Figure 7. Up-regulation of hypoxia markers and increased angiotensin II levels in the absence of ACE2

a,b, Northern blot analysis of BNIP3 and PAI-1 mRNA expression levels, two hypoxia-inducible genes in 6 month old male ace2$^{+/y}$ (n=5) and ace2$^{-/y}$ (n=5) mice. (a) shows individual Northern blot data and (b) relative levels of BNIP3 and PAI-1 mRNA levels normalized to the gapdh control. ·· p<0.01.

c, AngiotensinI (AngI) and AngiotensinII (AngII) peptide levels in the heart and kidneys of 6 month old male ace2$^{+/y}$ ( n = 8) and ace2$^{-/y}$ (n = 8) littermate mice. AngI and AngII tissue levels were determined by radioimmunoassays. Mean peptide levels +/- SD are shown. ·· p<0.01.

Figure 8. ACE-ACE2 double mutant mice do not develop heart failure

a, Blood pressure measurements in 6 month old male ace2$^{+/y}$ (n = 8), ace$^{-/-}$ (n = 8), and ace$^{-/-}$ ace2$^{-/y}$ double mutant (n = 6) mice. Mean values +/- SD are shown. Blood pressures were confirmed using invasive hemodynamic measurements. ·· p<0.01 of mutant as compared to wild type mice. b, Percentage fractional shorting and velocity of circumferential fiber shortening in 6 month old male ace2$^{+/y}$ (n = 8), ace2$^{-/y}$ ( n = 8), ace$^{-/-}$ (n = 8) and ace$^{-/-}$ ace2$^{-/y}$ double mutant (n = 6) littermates. Values were determined by echocardiography. Mean values +/- SD are shown. ·· p < 0.01 between genetic groups. The experimental data can be seen in Table 3. c, Echocardiographic measurements of contracting hearts in a 6 month old male ace2$^{+/y}$, ace2$^{-/y}$, ace$^{-/-}$, and ace$^{-/-}$ ace2$^{-/y}$ double mutant littermate mice. Echocardiograms were analyzed and are labeled as described in Figure 6a. Note the ablation of ACE in an ace2 null background completely rescues the contractile heart defects observed in ace2$^{-/y}$ single mutant mice.

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Figure 9. Percentage change in elastance from baseline was calculated over time. The lung elastance was calculated by dividing tracheal peek pressure with volume.

Figure 10. *a*, Human ACE2 DNA (SEQ ID NO:1). *b*, Human ACE2 polypeptide (SEQ ID NO:2).

Figure 11. *a*, Mouse ACE2 DNA (SEQ ID NO:3). *b*, Mouse ACE2 polypeptide (SEQ ID NO:4)

Figure 12. ACE2 nucleotide polymorphisms and sequences.

## DETAILED DESCRIPTION OF THE INVENTION

The invention provides a new paradigm for the regulation of the RAS system and shows that ACE2 is a critical negative regulator of the RAS required for heart function and cardiovascular function, kidney function and lung injury. Activation of ACE2 is critical for treatment and prevention of heart and kidney disease and hypertension and lung diseases. The invention teaches for the first time that administering an ACE2 activator to an animal prevents and treats heart failure and hypertension, kidney disease and lung injury.

This result is completely unexpected in view of prior art references, such as US Patent No. 6,194,556 and Canadian patent application no. 2,372,387, described above, that teach that ACE2 activity must be *inhibited* in order to treat heart disease. It is thus surprising that heart disease is actually treated by *activating* ACE2 expression and/or activity.

The invention includes activators that include but are not limited to activators of ACE2 function and/or ACE2 mRNA and ACE2 protein expression, and pharmaceutical compositions including the activators. The invention also includes methods of medical treatment of heart disease, lung disease and kidney disease and hypertension by administration of an effective amount of an activator to an animal in need of treatment. Lung disease includes but is not limited to chronic obstructive pulmonary disease, pneumonia, asthma,

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chromic bronchitis, pulmonary emphysema, cystic fibrosis, interstitial lung disease, primiary pulmonary hypertension, pulmonary embolism, pulmonary sarcodosis, tuberculosis and lung cancers.

The invention also includes screening assays for detecting ACE2 activators, which may be used to treat heart disease and kidney disease and hypertension and lung disease. These assays are *in vitro* or *in vivo*. In a preferred embodiment, the invention includes an endothelial, kidney, lung or heart cell assay for evaluating whether a candidate compound is capable of increasing ACE2 expression or activity. Cells are cultured in the presence of at least one compound whose ability to activate expression or activity is sought to be determined and the cells are measured for an increase in the level of ACE2 expression. Another aspect of the invention involves an ACE2 knock-out mouse for identifying compounds that may overcome the effects of loss of ACE2. Polypeptides and small organic molecules are tested in these assays. The invention includes all compounds that are identified with the screening methods of the invention and which are suitable for administration to animals in pharmaceutical compositions.

Another aspect of the invention is the diagnosis of the onset or risk of heart and/or kidney disease and/or hypertension and/or lung disease. This may be diagnosed by measuring ACE2 levels in heart, serum, or kidney, or other tissues. Levels of ACE2 less than wild type levels are indicative of an "ACE2 decreased state" which this invention shows is directly connected with heart and/or kidney disease and/or hypertension, and/or lung disease, or a risk of disease. Wild type levels of ACE2 and decreased levels will be readily apparent to those skilled in the art. An ACE2 decreased state is also indicated by the polymorphisms ACE2a-ACE2m described below. The invention is useful to treat and diagnose diseases associated with decreased ACE2 expression or activity. Diagnosis is also optionally accomplished by analysis of polymorphisms upstream and downstream of and within the ACE2 gene which are associated with an ACE2 reduced state. All the reagents required for the detection of nucleotide(s) that distinguish the polymorphisms,

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by means described herein, can be provided in a single kit for analysis of isolated genomic DNA from an animal. The kit would contain labelled probes that distinguish polymorphisms of ACE2 in order to allow genotyping and phenotyping, for diagnosis of risk or onset of disease. Polymorphism-specific

5      probes can be appropriately labelled and added to the generated DNA segments under annealing conditions, such that only one of the polymorphism-specific probes hybridizes and can be detected, thereby identifying the specific ACE2 polymorphism.

**Therapeutic Methods**

10     As hereinbefore mentioned, the present inventors have shown that ACE2 gene expression is down-regulated in hypertension, heart and kidney disease. Accordingly, the present invention provides a method of treating or preventing hypertension, heart disease, lung or kidney disease comprising administering an effective amount of an agent that can increase the expression of ACE2 to

15     an animal in need thereof.

The term "an agent that can increase the expression of ACE2" as used herein means any agent that can increase the level or activity of an ACE2 gene or protein as compared to the level or activity of the ACE2 gene or protein in the same type of cell in the absence of the agent. The agent can be any type of

20     substance including, but not limited to, nucleic acid molecules (including ACE2 or fragments thereof), proteins (including Ace2 or fragments thereof), peptides, carbohydrates, small molecules, or organic compounds. Whether or not the ACE2 gene is increased can be readily determined by one of skill in the art using known methods including Western blotting SDS-PAGE,

25     immunochemistry, RT-PCR, Northern blotting and in situ hybridization.

The term "animal" as used herein includes all members of the animal kingdom. The animals are preferably human.

The term "effective amount" as used herein means an amount effective at dosages and for periods of time necessary to enhance the level of ACE2.

The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent

5     of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

10     *Administration of ACE2 nucleic acid molecule*

In one embodiment, the expression of the ACE2 gene is increased by administering a nucleic acid that comprises an ACE2 gene or portion thereof.

In another embodiment, the expression of the ACE2 gene may be increased by administering an agent that increases ACE2 gene expression including any

15     agents identified using the screening assays in this application.

Since an animal suffering from disease, disorder or abnormal physical state can be treated by up regulation of ACE2, gene therapy to increase ACE2 expression is useful to modify the development/progression of heart or kidney or lung disease.

20     The invention includes methods and compositions for providing ACE2 gene therapy for treatment of diseases, disorders or abnormal physical states characterized by decreased ACE2 expression or levels of activity of ACE2 polypeptide.

The invention includes methods and compositions for providing a nucleic acid

25     molecule encoding ACE2 or functionally equivalent nucleic acid molecule to the cells of an animal such that expression of ACE2 in the cells provides the biological activity or phenotype of ACE2 polypeptide to those cells. Sufficient amounts of the nucleic acid molecule are administered and expressed at sufficient levels to provide the biological activity or phenotype of ACE2

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polypeptide to the cells. For example, the method can preferably involve a method of delivering a nucleic acid molecule encoding ACE2 to the cells of an animal having cardiovascular or kidney, or lung disease, comprising administering to the subject a vector comprising DNA encoding ACE2. The
5    method may also relate to a method for providing an animal having cardiovascular or kidney, or lung disease with biologically active ACE2 polypeptide by administering DNA encoding ACE2. The method may be performed *in vivo* or *ex vivo* (e.g. with heart, lung, endothelial or kidney stem cells, progenitor cells or other cells to be transplanted cells). Methods and
10   compositions for administering ACE2 (including in gene therapy) to isolated cell or an animal are explained, for example, in U.S. Patent Nos. 5,672,344, 5,645,829, 5,741,486, 5,656,465, 5,547,932, 5,529,774, 5,436,146, 5,399,346, 5,670,488, 5,240,84, 6,322,536, 6,306,830 and 6,071,890 and US Patent Application No. 20010029040 which are incorporated by reference in
15   their entirety.

The method also relates to a method for producing a stock of recombinant virus by producing virus suitable for gene therapy comprising DNA encoding ACE2. This method preferably involves transfecting cells permissive for virus replication (the virus containing the nucleic acid molecule) and collecting the
20   virus produced.

The methods and compositions can be used *in vivo* or *in vitro*. The invention also includes compositions (preferably pharmaceutical compositions for gene therapy). The compositions include a vector containing ACE2. The carrier may be a pharmaceutical carrier or a host cell transformant including the
25   vector. Vectors known in the art include but are not restricted to retroviruses, adenoviruses, adeno associated virus (AAV), herpes virus vectors, such as vaccinia virus vectors, HIV and lentivirus-based vectors, or plasmids. The invention also includes packaging and helper cell lines that are required to produce the vector. Methods of producing the vector and methods of gene
30   therapy using the vector are also included with the invention.

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The invention also includes a transformed cell containing the vector and the recombinant ACE2 nucleic acid molecule sequences.

5       *Use of ACE2 Variants* - Modifications to polypeptide sequence

ACE2 variants may be used in methods of the invention. Changes which result in production of a chemically equivalent or chemically similar amino acid sequence are included within the scope of the invention. Polypeptides having sequence identity to ACE2 receptor are tested to ensure that they are suitable

10      for use in the methods of the invention. Variants of the polypeptides of the invention may occur naturally, for example, by mutation, or may be made, for example, with polypeptide engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for

15      another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as

20      arginine.

Therefore, the invention includes polypeptides having conservative changes or substitutions in amino acid sequences. Conservative substitutions insert one or more amino acids, which have similar chemical properties as the replaced amino acids. The invention includes sequences where conservative

25      substitutions are made that do not destroy compound activity.

Polypeptides comprising one or more d-amino acids are contemplated within the invention. Also contemplated are polypeptides where one or more amino acids are acetylated at the N-terminus. Those with skill in the art recognize that a variety of techniques are available for constructing polypeptide

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mimetics with the same or similar desired compound activity as the corresponding polypeptide compound of the invention but with more favorable activity than the polypeptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis.  See, for example, Morgan and
5    Gainor, Ann. Rep. Med. Chem., 24:243-252 (1989).  Examples of polypeptide mimetics are described in U.S. Patent Nos. 5,643,873. Other patents describing how to make and use mimetics include, for example in, 5,786,322, 5,767,075, 5,763,571, 5,753,226, 5,683,983, 5,677,280, 5,672,584, 5,668,110, 5,654,276, 5,643,873.  Mimetics of the polypeptides of the
10   invention may also be made according to other techniques known in the art. For example, by treating a polypeptide of the invention with an agent that chemically alters a side group by converting a hydrogen group to another group such as a hydroxy or amino group.  Mimetics preferably include sequences that are either entirely made of amino acids or sequences that are
15   hybrids including amino acids and modified amino acids or other organic molecules.

The invention also includes hybrids and polypeptides, for example where a nucleotide sequence is combined with a second sequence.

The invention also includes methods of using polypeptide fragments of ACE2
20   which may be used to confer compound activity if the fragments retain activity. The invention also includes polypeptides and fragments of the polypeptides of the invention which may be used as a research tool to characterize the polypeptide or its activity.  Such polypeptides preferably consist of at least 5 amino acids.  In preferred embodiments, they may consist of 6 to 10, 11 to 15,
25   16 to 25, 26 to 50, 51 to 75, 76 to 100 or 101 to 250 or 250 to 500 amino acids.  Fragments may include sequences with one or more amino acids removed, for example, C-terminus amino acids in a compound sequence.

*Enhancement of ACE2 polypeptide activity*

The activity of ACE2 is increased or decreased by carrying out selective site-
30   directed mutagenesis.  A DNA plasmid or expression vector containing the

nucleic acid molecule or a nucleic acid molecule having sequence identity is preferably used for these studies using the U.S.E. (Unique site elimination) mutagenesis kit from Pharmacia Biotech or other mutagenesis kits that are commercially available, or using PCR. Once the mutation is created and confirmed by DNA sequence analysis, the mutant polypeptide is expressed using an expression system and its activity is monitored.

The invention also includes methods of use of polypeptides which have sequence identity at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to human or mouse ACE2 (or a partial sequence thereof). Modified polypeptide molecules are discussed below. Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above). BLAST is a series of programs that are available online at http://www.ncbi.nlm.nih.gov/BLAST. The advanced BLAST search (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1) is set to default parameters. (i.e. Matrix BLOSUM62; Gap existence cost 11; Per residue gap cost 1; Lambda ratio 0.85 default).

References to BLAST searches are: Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410; Gish, W. & States, D.J. (1993) "Identification of protein coding regions by database similarity search." Nature Genet. 3:266-272; Madden, T.L., Tatusov, R.L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131-141; Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389-3402; Zhang, J. & Madden, T.L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." Genome Res. 7:649-656.

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Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified. The invention includes polypeptides with mutations that cause an amino acid change in a portion of the polypeptide not involved in providing activity or an amino acid change in a

5    portion of the polypeptide involved in providing activity so that the mutation increases or decreases the activity of the polypeptide.

**Screening for ACE2 activators**

Small organic molecules are screened to determine if they increase ACE2 expression or activity. Polypeptide fragments of ACE2 as well as polypeptides

10   having sequence identity to ACE2 are also tested to determine if they increase ACE2 activity *in vitro* assays and *in vivo* in cell lines. Activators are preferably directed towards specific domains of ACE2 to increase ACE2 activation. To achieve specificity, activators should target the unique sequences of ACE2.

15   The present invention also includes the isolation of substances that increase ACE2 expression. In particular ligands or substances that can bind to the ACE2 gene or protein may be isolated. Biological samples and commercially available libraries may be tested for substances such as proteins that bind to a ACE2 gene or protein. For example, the amino acid sequence of a ACE2

20   protein may be used to probe peptide libraries while a nucleic acid sequence encoding ACE2 may be used to probe nucleic acid libraries. In addition, antibodies prepared to ACE2 may be used to isolate other peptides with affinity for ACE2. For example, labelled antibodies may be used to probe phage displays libraries or biological samples.

25   Conditions which permit the formation of complexes with a substance and a ACE2 gene or protein may be selected having regard to factors such as the nature and amounts of the substance and the ACE2 gene or protein. The substance-protein or substance-gene complex, free substance or non-complexed substance may be isolated by conventional isolation techniques,

30   for example, salting out, chromatography, electrophoresis, gel filtration,

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fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against ACE2 or the substance, or labelled protein, or a labelled substance may be utilized. The antibodies, proteins, or substances may be labelled, as

5   appropriate, with a detectable substance as described below.

Once potential binding partners have been isolated, screening methods may be designed in order to determine if the substances that bind to the ACE2 genes or proteins are useful in the methods of the present invention to enhance ACE2 expression on a cell and therefore useful in treating disease.

10   Therefore, the invention also provides methods for identifying substances which are capable of binding to ACE2 genes or proteins. In particular, the methods may be used to identify substances, which are capable of binding to and augmenting or enhancing expression of the ACE2. Accordingly the invention provides a method of identifying substances, which bind with a

15   ACE2 gene or protein comprising the steps of:

(a) reacting a ACE2 gene or protein, preferably immobilized, and a test substance, under conditions which allow for formation of a complex, and

(b) assaying for complexes, for free substance, and for non-

20   complexed gene or protein.

Any assay system or testing method that detects protein-protein interactions may be used including co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Additionally, x-ray crystallographic studies may be used as a means of evaluating interactions

25   with substances and molecules. For example, purified recombinant molecules in a complex of the invention when crystallized in a suitable form are amenable to detection of intra-molecular interactions by x-ray crystallography. Spectroscopy may also be used to detect interactions and in particular, Q-TOF instrumentation may be used. Biological samples and

30   commercially available libraries may be tested for ACE2 binding peptides. In

addition, antibodies prepared to the ACE2 may be used to isolate other peptides with ACE2 binding affinity. For example, labelled antibodies may be used to probe phage display libraries or biological samples. In this respect peptides may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate expression vectors. (see Christian *et al.* 1992, J. Mol. Biol. 227:711; Devlin *et al.*, 1990 Science 249:404; Cwirla *et al.* 1990, Proc. Natl. Acad, Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping peptides (see U.S. Pat. No. 4,708,871). Activators are tested in hypertension, heart or kidney disease model animals.

In one embodiment, the invention includes an assay for evaluating whether a candidate compound is capable of increasing ACE2 expression or activity by culturing cells (preferably kidney or heart cells) in the presence of at least one compound whose ability to activate expression or activity is sought to be determined and thereafter monitoring the cells for an increase in the level of ACE2 expression and/or activity. Increased ACE2 expression and/or activity indicates that the candidate compound is useful for treating heart or kidney disease or hypertension.

A similar screening assay may be done with mammals known in the art that are prone to heart disease or kidney disease. The candidate compound is administered to the mammal and ACE2 expression and/or activity is measured. Increased ACE2 expression and/or activity indicates that the candidate compound is useful for treating heart or kidney disease.

A method of determining whether a candidate compound increases the activity of ACE2 (and is useful for treating cardiovascular disease and/or kidney disease and/or lung, and/or hypertension) can also include:

a)      contacting (i) ACE2, a fragment of ACE2 or a derivative of either of the foregoing with (ii) an ACE2 substrate in the presence of the candidate compound; and

b)      determining whether ACE2 activity on the substrate is increased, thereby indicating that the compound increases the activity of ACE2. Increased ACE2 activity indicates that the compound is useful for treating heart diseases or kidney or lung diseases listed in this application or hypertension.  Determination of an increase in ACE2 activity preferably involves determining whether the compound increases ACE2 proteolytic activity (increased hydrolysis of substrates).  In a variation of the invention, the assays of the invention are subject to the proviso that the candidate compound is not a sodium-halogen salt or a potassium-halogen salt and the assay is not directed to measuring the effect of increasing ion concentration on ACE2 proteolysis.  ACE2 substrates include AngI, AngII, des-Ang, AngII, apelin-13, dynorphin 13, beta-casomorphin and neurotensin.

Methods for producing ACE2 are described in CA 2,372,387.  An example of an *in vitro* assay for ACE2 activation is shown in Vickers *et al.,* Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase.  J Biol. Chem. 2002, 277(17):14838.  Other assays (as well as variations of the above assays) will be apparent from the description of this invention and techniques such as those disclosed in U.S. Patent Nos. 5,851,788, 5,736,337 and 5,767,075 which are incorporated by reference in their entirety.

**Knock-Out Mammals**

Working examples of the cloning of mouse ACE2 and generation of ACE2 knock-out mice are described in the examples below.  The term "knockout" refers to partial or complete reduction of the expression of at least a portion of a polypeptide encoded by an ACE2 gene of a single cell, selected cells, or all of the cells of a mammal.  The mammal may be a "heterozygous knockout", wherein one allele of the endogenous gene has been disrupted and one allele

still exists. In ACE2 on the X chromosome, females may be heterozygous. In males, there is only one allele and males are homozygous. Alternatively, the mammal may be a "homozygous knockout" wherein both alleles of the endogenous gene have been disrupted.

5     The term "knockout construct" refers to a nucleotide sequence that is designed to decrease or suppress expression of a polypeptide encoded by an endogenous gene in one or more cells of a mammal. The nucleotide sequence used as the knockout construct is typically comprised of (1) DNA from some portion of the endogenous gene (one or more exon sequences, 10     intron sequences, and /or promoter sequences) to be suppressed and (2) a marker sequence used to detect the presence of the knockout construct in the cell. The knockout construct is inserted into a cell containing the endogenous gene to be knocked out. The knockout construct can then integrate within one or both alleles of the endogenous ACE2 gene, and such integration of the 15    ACE2 knockout construct can prevent or interrupt transcription of the full-length endogenous ACE2 gene. Integration of the ACE2 knockout construct into the cellular chromosomal DNA is typically accomplished via homologous recombination (i.e., regions of the ACE2 knockout construct that are homologous or complimentary to endogenous ACE2 DNA sequences can 20    hybridize to each other when the knockout construct is inserted into the cell; these regions can then recombine so that the knockout construct is incorporated into the corresponding position of the endogenous DNA).

Typically, the knockout construct is inserted into an undifferentiated cell termed an embryonic stem cell (ES cell). ES cells are usually derived from an 25    embryo or blastocyst of the same species as the developing embryo into which it can be introduced, as discussed below.

The phrases "disruption of the gene", "gene disruption", "suppressing expression", and "gene suppression", refer to insertion of an ACE2 nucleotide sequence knockout construct into a homologous region of the coding region 30    of the endogenous ACE2 gene (usually containing one or more exons) and/or the promoter region of this gene so to decrease or prevent expression of the

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full length ACE2 molecule in the cell. Insertion is usually accomplished by homologous recombination. By way of example, a nucleotide sequence knockout construct can be prepared by inserting a nucleotide sequence comprising an antibiotic resistance gene into a portion of an isolated

5     nucleotide sequence encoding ACE2 that is to be disrupted. When this knockout construct is then inserted into an embryonic stem cell ("ES cell"), the construct can integrate into the genomic DNA of at least one ACE2 allele. Thus, many progeny of the cell will no longer express ACE2 at least in some cells, or will express it at a decreased level and/or in a truncated form, as at

10    least part of the endogenous coding region of ACE2 is now disrupted by the antibiotic resistance gene.

The term "marker sequence" refers to a nucleotide sequence that is (1) used as part of a larger nucleotide sequence construct (i.e., the "knockout construct") to disrupt the expression of ACE2 and (2) used as a means to

15    identify those cells that have incorporated the ACE2 knockout construct into the chromosomal DNA. The marker sequence may be any sequence that serves these purposes, although typically it will be a sequence encoding a protein that confers a detectable trait on the cell, such as an antibiotic resistance gene or an assayable enzyme not naturally found in the cell. The

20    marker sequence will also typically contain either a homologous or heterologous promoter that regulates its expression.

Included within the scope of this invention is a mammal in which one or both ACE2 alleles, as well as one or both alleles of another gene(s), have been knocked out. Such a mammal can be generated by repeating the procedures

25    set forth herein for generating an ACE2 knockout mammal but using another gene, or by breeding two mammals, one with one or both alleles of ACE2 knocked out, and one with one or both alleles of a second gene knocked out, to each other, and screening for those offspring that have the double knockout genotype (whether a double heterozygous or a double homozygous knockout

30    genotype, or a variation thereof).

Other knock out animals and cells may be made using similar techniques.

**Pharmaceutical compositions**

Activators of ACE2 expression and activity are preferably combined with other components, such as a carrier, in a pharmaceutical composition. These compositions may be administered to an animal, preferably a human, in
5   soluble form to prevent or treat heart disease, kidney disease or hypertension. Heart diseases include chronic heart failure, left ventricular hypertrophy, acute heart failure, myocardial infarction, and cardiomyopathy. Kidney disease includes kidney failure. ACE2 activators are useful for regulating blood pressure and arterial hypertension. Normal blood pressure has a diastolic
10  blood pressure of less than 85 mm Hg. High normal blood pressure has a diastolic blood pressure between 85 and 89 mm Hg. Mild hypertension corresponds to a diastolic blood pressure between 90-104 mm Hg. Moderate hypertension has to a diastolic blood pressure between 105 and 114 mm Hg. Severe hypertension has a diastolic blood pressure higher than 115 mm Hg.
15  Abnormal blood pressure is also determined from the systolic blood pressure (when the diastolic pressure is less than 90 mm Hg). Normal blood pressure has a systolic blood pressure of less than 140 mm Hg. Borderline systolic hypertension shows a systolic blood pressure between 140 and 159 mm Hg. Isolated systolic hypertension has a systolic blood pressure higher than 160
20  mm Hg. (Cecil: Essentials of Medicine, Third Edition by Andreoli et al. W.B. Saunders Company (1993)). Hypertension is diagnosed in an adult over 18 years old if the average of two or more blood pressure measurements on at least two visits is 90 mm Hg or higher diastolic or 140 mm Hg systolic. Children and pregnant women have a lower blood pressure, so a blood
25  pressure over 120/80 (i.e., 120 mm Hg systolic blood pressure/80 mm Hg diastolic blood pressure) indicates hypertension.

The pharmaceutical compositions can be administered to humans or animals by a variety of methods including, but not restricted to topical administration, oral administration, aerosol administration, intratracheal instillation, intraperitoneal
30  injection, and intravenous injection. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration.

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Polypeptides may be introduced into cells using *in vivo* delivery vehicles such as but not exclusive to liposomes.

The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be
5   administered to patients, such that an effective quantity of the nucleic acid molecule or polypeptide is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA).

10  On this basis, the pharmaceutical compositions could include an active compound or substance, such as a nucleic acid molecule or polypeptide, in association with one or more pharmaceutically acceptable vehicle or diluent, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the active molecules with the
15  vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within tissue.

**Heterologous overexpression of ACE2**

Expression vectors are useful to provide high levels of ACE2 expression. Cell
20  cultures transformed with the nucleic acid molecules of the invention are useful as research tools, particularly for studies of ACE2 decreased states. The invention includes vectors selective for heart cells and kidney cells preferably endothelial cells which normally make ACE2. The invention also includes transfected cells including these vectors. Examples of vectors for
25  heart and kidney cells are described, for example, in Rosengart *et al.* US Patent No. 6,322,536; March *et al.* US Patent No. 6,224,584; Hammond *et al.* US Patent No. 6,174,871; Wolfgang-M. Franz *et al.* Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promoters. Cardiovascular Research 35(1997) 560-566; Rothmann T. *et al.*
30  Heart muscle-specific gene expression using replication defective

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recombinant adenovirus. Gene Ther 1996 Oct;3(10):919-26; Phillips MI *et al.*
Vigilant vector: heart-specific promoter in an adeno-associated virus vector for
cardioprotection. Hypertension 2002, Feb; 39(2 Pt 2):651-5; Herold BC *et al.*
Herpes simplex virus as a model vector system for gene therapy in renal
5    disease. Kidney Int 2002 Jan;61 Suppl 1:3-8; Figlin RA *et al.* Technology
evaluation: interleukin-2 gene therapy for the treatment of renal cell
carcinoma. Curr Opin Mol Ther 1999 Apr;1(2):271-8; Varda-Bloom N *et al.*
Tissue-specific gene therapy directed to tumor angiogenesis. Gene Ther 2001
Jun;8(11):819-27; Scott-Taylor TH *et al.*. Adenovirus facilitated infection of
10   human cells with ecotropic retrovirus. Gene Ther 1998 May;5(5):621-9;
Langer JC *et al.* Adeno-associated virus gene transfer into renal cells:
potential for *in vivo* gene delivery. Exp Nephrol 1998 May-Jun;6(3):189-94;
Lien YH *et al.* Gene therapy for renal diseases. Kidney Int Suppl 1997
Oct;61:S85-8; and Ohno K *et al.* Cell-specific targeting of Sindbis virus
15   vectors displaying IgG-binding domains of protein A. Nat Biotechnol 1997
Aug;15(8):763-7.

Cell cultures, preferably heart and kidney cell cultures and endothelial cell
cultures, are used in overexpression and research according to numerous
techniques known in the art.  For example, a cell line (either an immortalized
20   cell culture or a primary cell culture) may be transfected with a vector
﹀ containing a ACE2  nucleic acid molecule (or molecule having sequence
identity) to measure levels of expression of the nucleic acid molecule and the
activity of the nucleic acid molecule and polypeptide.  The cells are also useful
to identify compounds that bind to and activate the polypeptide.

25   **Diagnostic Kits Measuring ACE2 Activity and/or Expression**

The measurement of ACE2 expression or activity is also used in: i) diagnosis
of heart or kidney disease, lung disease and/or hypertension ii) identifying
patients at risk of developing such disease prior to the development of
disease iii) measuring therapeutic response in patients having heart disease
30   such as coronary artery disease, chronic heart failure, or kidney disease
and/or hypertension and/or lung injury and iv). measuring the success of

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interventional disease preventive strategies in such patients at risk. The invention includes a method for assessing the levels of ACE2 in an animal comprising the following steps: (a) preparing a heart or kidney or lung sample from a specimen collected from the animal; (b) testing for the presence of ACE2 in the sample; and (c) correlating the presence or levels of ACE2 in the sample with the presence (or risk) of disease such as heart or kidney or lung disease in the animal. ACE2 levels below normal or low ACE2 activity indicate the presence or risk of disease.

**Diagnostic Kits based on ACE2 single nucleotide polymorphisms**

This invention also shows that decreased human ACE2 expression results from polymorphisms that control ACE2 gene expression. The QTL mappings in the rats show that there is a 100% correlation between reduced expression levels of ACE2 and hypertension and cardiovascular and kidney disease. None of the polymorphisms described below are found within the ACE2 coding region. All are upstream or downstream of the ACE2 coding region. None of these polymorphisms or their role in cardiovascular disease, kidney disease, lung disease and hypertension were previously known. A particular SNP haplotype is associated with increased risk of disease. This haplotype is an important diagnostic tool for the assessment of risk of disease and for the determination of appropriate medical treatment.

The polymorphisms are as follows:

| SNP name | SNP description | African Am | Asian | Caucasian | Reference |
|----------|-----------------|------------|-------|-----------|-----------|
| ACE2a rs879922 | C(C/G) | 60 | 100 | 70 | C |
| ACE2b rs757066 | T(C/T) | 100 | 100 | 70 | T |
| ACE2c rs714205 | C(C/G) | 70 | 50 | 80 | C |
| ACE2d rs329442 | C(A/C) | 50 | 90 | 90 | A/C |
| ACE2e rs233574 | C(C/T) | 80 | 100 | 60 | C |
| ACE2f rs1978124 | C(C/T) | 90 | 100 | 50 | C |
| ACE2g rs1514282 | A(A/G) | 70 | 100 | 100 | A |
| ACE2h | A(A/G) | 20 | 50 | 30 | A |

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| rs1514282-2 | | | | | |
|---|---|---|---|---|---|
| ACE2i | A(A/G) | 70 | 100 | 100 | A |
| rs1514281 | | | | | |
| ACE2j | A(A/G) | 20 | 50 | 50 | A |
| rs1514281-2 | | | | | |
| ACE2k | A(A/G) | | 100 | 70 | A |
| rs1514279 | | Failed | | | |
| ACEl | C(C/T) | 80 | 100 | 80 | C |
| 2 rs1514280 | | | | | |
| ACE2m | C(C/T) | 100 | 100 | 50 | C |
| rs233575 | | | | | |

The nucleotide number of the polymorphisms that control ACE2 gene expression, as described in the chart above and in Figure 11, can be readily determined by a person skilled in the art.

The chart shows the percentage of the reference base found in each of the
5    three populations in the chart (African American, Caucasian, Asian). For example, for SNP rs233574, the predicted SNP is C/T, with the reference peak being C. In this case, the African American allele frequency is 80% C; the Asian allele frequency is 100% C (in other words, a monomorphic marker); and the Caucasian allele frequency is 60% C.

10   The present invention provides polynucleotide probes which can be used to determine an animal's genotype which is whether a person is homozygous for one or the other of the polymorphisms, or heterozygous for these polymorphisms, and by extension, the person's phenotype. The phenotype indicates the amount of ACE2 expression in the person's cells. Further, the
15   invention provides methods of using such polynucleotides in such genotype and phenotype determinations. The oligonucleotides of the invention can be used as probes to detect nucleic acid molecules according to techniques known in the art (for example, see US Patent Nos. 5,792,851 and 5,851,788).

For example, a polynucleotide of the invention may be converted to a probe
20   by being end-labelled using digoxigenin-11-deoxyuridine triphosphate. Such probes    may    be    detected    immunologically    using alkaline-phosphate-conjugated polyclonal sheep antidigoxigenin F(ab) fragments and nitro blue tetrazolium with 5-bromo-4-chloro-3-indoyl phosphate as chromogenic substrate.

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Thus, in accordance with one aspect of the present invention, a polynucleotide probe is provided that selectively hybridizes to a portion of the ACE upstream or downstream sequence. A probe may be designed to hybridise to one ACE2 polymorphism under stringent conditions but not the

5      other polymorphisms in order to distinguish a particular polymorphism.

The polymorphism-specific polynucleotide hybridization probes of the invention may comprise, for example, genomic DNA or synthetic DNA. Such oligonucleotide probes can be synthesised by automated synthesis and will preferably contain about 10 - 30 bases, although as understood in the

10     oligonucleotide probe hybridization assay art, as few as 8 and as many as about 50 nucleotides may be useful, depending on the position within the probe where the potential mismatch with the target is located, the extent to which a label on the probe might interfere with hybridization, and the physical conditions (e.g., temperature, pH, ionic strength) under which the

15     hybridization of probe with target is carried out. In accordance with conventional procedures, the design of a polynucleotide probe according to the present invention preferably involves adjusting probe length to accommodate hybridization conditions (temperature, ionic strength, exposure time) while assuring polymorphism-specificity.

20     In accordance with another aspect of the present invention, a test kit for genotyping is provided comprising:

(a) means for amplifying nucleic acid that comprises at least a portion of an ACE2 5' or 3' region, wherein the portion includes a nucleotide corresponding to one of ACE2a-ACE2m; and

25     (b) a polynucleotide probe of the invention, that distinguishes one ACE2 polymorphism from the other.

The "means for amplifying" will, as the skilled will readily understand, depend on the amplification method to be used. Thus, for example, these means might include suitable primers, a suitable DNA polymerase, and the four

30     2'-deoxyribonucleoside triphosphates (dA, dC, dG, dT), if amplification is to be

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by the PCR method. To cite another example, if the amplification is to be by a method relying on transcription, such as the 3SR method, the means will include two primers, at least one of which, when made double-stranded, will provide a promoter, an RNA polymerase capable of transcribing from that

5    promoter, a reverse transcriptase to function in primer-initiated, DNA-directed and RNA-directed, DNA polymerization and possibly also in RNAse H degradation of RNA to free DNA strands from RNA/RNA hybrids, the four ribonucleoside triphosphates (A, C, G and U), and the four 2'-deoxyribonucleoside triphosphates. In another example, if the amplification

10   is by the ligase chain reaction, the means will include two oligonucleotides (DNAs) and a suitable DNA ligase that will join the two if a target, to which both can hybridize adjacent to one another in ligatable orientation, is present.

The oligonucleotide probes of the invention will preferably be labelled. The label may be any of the various labels available in the art for such probes,

15   including, but not limited to $^{32}$P; $^{35}$S; biotin (to which a signal generating moiety, bound to or complexed with avidin can be complexed); a fluorescent moiety; an enzyme such as alkaline phosphatase (which is capable of catalysing a chromogenic reaction); digoxigenin, as described above; or the like.

20   RFLP analysis, electrophoretic SSCP analysis or sequencing analysis may also be used to detect an ACE2 polymorphism.

There has also been provided, in accordance with another aspect of the present invention, a method of typing for an ACE2 polymorphism-specific target sequence in a ACE2 nucleic acid derived from an animal, comprising

25   the steps of,

(a) obtaining, by a target nucleic acid amplification process applied to DNA from heart or kidney, an assayable quantity of amplified nucleic acid with a sequence that is that of a subsequence (or the complement of a subsequence) of an upstream or downstream region of ACE2, said

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subsequence including a nucleotide where an ACE2 polymorphism may occur; and

(b) analyzing (e.g., in a nucleic acid probe hybridization assay employing a polynucleotide probe according to the invention) the amplified nucleic acid
5       obtained in step (a) to determine the base or bases at the polymorphism position.

In one application of the typing methods of the invention, the methods are applied to an individual to determine whether the individual is at risk of developing heart or kidney disease.

10    People with coronary artery disease and/or following bypass surgery, have cardiac hypoxia. It is also known as cardiac stunning or cardiac hibernation. These patients display little structural changes in the heart but have reduced heart function. It is very uncommom to have altered heart function in the absence of structural changes. In mouse models of cardiac stunning or
15    hypoxia, the animals have a phenotype that precisely resemble that of ACE2 mice. In addition, we have shown that markers of hypoxia are induced in the ACE2 deficient mice. Taken together our data show that these mice have reduced heart function due to chronic hypoxia, and thus are models of coronary artery disease. Thus, the polymorphisms and/or reduced ACE2
20    expression or activity may be used to diagnose this state in humans. Another example is to test whether the function of the heart in patients with dilated cardiomyopathy and show that the disease outcome is associated with ACE2 polymorphisms. Increasing ACE2 expression and activity may be used to treat this state.

25    **Characterization of ACE2 as a Negative Regulator of the RAS**

ACE2 maps to a QTL associated with hypertension in three rat models of high blood pressure and ACE2 levels are reduced in all of these hypertensive rat strains. In mice, genetic inactivation of ACE2 using homologous recombination results in increased AngII peptide levels in tissues,
30    upregulation of hypoxia genes in heart, and severe cardiac dysfunction.

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Ablation of ACE expression on an ace2-deficient background completely abolished the heart failure phenotype of ace2 single knockout mice. These data provide a new paradigm for the regulation of the RAS and identify ACE2 as a negative regulator of the RAS that controls heart function.

5

### ACE2 and blood pressure control.

Most cardiovascular diseases are multifactorial quantitative traits controlled by both genetic and environmental factors. One major factor for cardiovascular
10    disease is the RAS. In contrast to ACE which is ubiquitously expressed, the recently identified ACE2 displays tissue-specific expression. ACE2 regulates endogenous AngII levels, by competing with ACE for its AngI substrate and/or by cleaving AngII to generate Ang1-7. Prior to this invention, nothing was known about the in vivo role of ACE2 in the cardiovascular system. ACE2
15    regulates endogenous levels of AngII. It also functions as a negative regulator of the RAS.

In three different rat strains that develop spontaneous or diet-induced hypertension and cardiovascular disease, ACE2 maps within a defined QTL on the X chromosome. In all of these hypertension susceptible rat strains,
20    ACE2 mRNA and protein levels were downregulated. The SS-X locus identified in QTL analysis in Sabra rats was also identified as a locus that confers resistance to salt loading. The reduction in ACE2 in the salt sensitive strain and the absence of any alteration in its expression in the resistant strain shows that ACE2 confers resistance to diet-induced blood pressure changes.

25    The map position and reduced expression show that ACE2 is the gene contributing to the hypertensive QTL on the X-chromosome. Moreover, increased AngI and AngII expression in ace2 null mice confirm that ACE2 is a regulator of the RAS system in vivo. However, loss of ACE2 in our mice did not result in any direct changes in blood pressure even when ACE function

was blocked. Blood pressure changes only occurred when extreme cardiac dysfunction was present in older male mice. The genetic factors that contribute to hypertension do not by themselves alter blood pressure. Rather, these QTL define single determinants of blood pressure, which in concert with other genetic polymorphisms promote the change in blood pressure. We identified the association of ACE2 polymorphisms with high blood pressure in the human population. Importantly, our data shows that ACE2 functions as a negative regulator of increased blood pressure.

**ACE2 and the control of heart function.**

Unexpectedly, loss of ACE2 in mice results in profound contractile dysfunction leading to severe reduction of systemic blood pressure in older mice. Importantly, this cardiac dysfunction is completely reversed by the disruption of ACE suggesting that a catalytic product of ACE triggers contractile impairment in the absence of ACE2. Since these contractility defects can occur in the absence of hypertrophy or any detectable changes in blood pressure, our data also provides genetic proof that the RAS regulated heart disease phenotype can be genetically uncoupled from its effects on blood pressure and cardiac hypertrophy.

ACE inhibitors and AngII receptor blockers have been shown to have a cardioprotective role in heart failure in humans, thus implicating AngII in cardiac disease. The complete abolition of the cardiac dysfunction in our *ace/ace2* double mutant mice shows that the RAS directly controls heart function and that ACE2 is a critical negative regulator that antagonizes the RAS and heart failure. Our genetic rescue experiments strongly indicate that it is in fact a product of ACE that drives heart failure, i.e., the increase in AngII seen in the hearts of *ace2* null mice is causative for cardiac dysfunction. Whether pharmacological inhibition of the AngII receptor rescues the heart phenotypes of *ace2* mutant mice needs to be determined. Interestingly, our results in flies show that a P-element mutation associated with the ACE homologue, ACER, results in a severe and lethal defect of heart

morphogenesis (data not shown) showing that the ACE/ACE2 functions in the heart have been conserved through evolution.

The defect in the *ace2* mutant hearts is characterized by severe contractile dysfunction and up-regulation of hypoxia-regulated genes with only slight 5    remodeling in older mice, no hypertrophy and no evidence of myocyte loss. These phenotypic and molecular parameters of failing hearts in *ace2* mutant mice are different from hypertrophy and dilated cardiomyopathy. Rather intriguingly, *ace2* mutant hearts resemble cardiac stunning and hibernation found in human cases of coronary artery disease and in cases of by-pass 10   surgery. In these human diseases and in animal models of cardiac stunning/hibernation, chronic hypoxic conditions lead to compensatory changes in myocyte metabolism, upregulation of hypoxia-induced genes, and reduced heart function. Since ACE2 is expressed in the vascular endothelium, and not cardiac myocytes, it is likely that the effects of ACE2 are confined to 15   the vasculature. For instance, local increases in AngII could lead to vasoconstriction resulting in hypoperfusion and hypoxia. AngII has also been shown to cause endothelial dysfunction via the induction of oxidative stress. The mechanisms by which loss of ACE2 can result in the upregulation of hypoxia-inducible genes needs to be determined. Importantly, our data show 20   that ACE2 polymorphisms cause the pathology of coronary heart disease in humans.

**EXAMPLES**

**ACE2 maps to a QTL on the X-chromosome in three hypertensive rat strains.**

25   Hypertension and most cardiovascular diseases are multifactorial in nature and disease pathogenesis is influenced by multiple genetic susceptibility loci. In various recombinant rat models, multiple QTL for hypertension have been identified. Since ACE2 maps to the X-chromosome in human and a QTL has been mapped to the X-chromosome in several rat models of hypertension 30   with no candidate gene ascribed to it as yet, ACE2 could be a candidate gene

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for this QTL. To facilitate chromosomal mapping of rat ACE2, the full-length rat ACE2 cDNA was cloned by screening a rat kidney cDNA library. Rat ACE2 is highly homologous to human ACE2 and is 32% identical and 42% similar to human and mouse ACE (Fig. 1a). Like human ACE2, the rat gene is

5   comprised of a single ACE domain with a conserved zinc binding site, a signal peptide and a transmembrane domain (Fig. 1b). Similar to human, ACE2 in mouse and rat is predominantly expressed in kidney and heart, with weaker expression in lung and liver (Fig. 1c).

Radiation hybrid mapping showed that the rat ACE2 gene maps on the X-

10   chromosome with significant LOD scores to markers *DXRat9, DXWox14, DXWox15 and DXRat42*, placing *ace2* between *DXRat9* and *DXRat42* (Fig. 1d). Comparative mapping showed that the *ace2* map position overlaps with a QTL interval for hypertension identified in Sabra salt-sensitive rats found between markers *DXMgh12* and *DXRat8* (*SS-X*). Moreover, the chromosomal

15   *ace2* region maps to the *B P3* QTL interval defined in stroke-prone spontaneously hypertensive rats (SHRSP) rats, and a previously identified hypertensive BB.Xs QTL identified on the X-chromosome of spontaneous hypertensive rats (SHR) by congenic analysis (Fig. 1d). Thus, ACE2 maps to a QTL on the rat X-chromosome identified in three separate models of

20   spontaneous and diet-induced hypertension.

**Downregulation of ACE2 expression in hypertensive rats**

Since the kidney is a major site of blood pressure regulation, ACE2 expression levels in the kidneys of these three hypertensive rat strains was determined. ACE2 mRNA levels were initially measured in the kidneys of salt-

25   sensitive Sabra hypertensive (SBH/y) rats and control salt-resistant Sabra normotensive (SBN/y) rats. Salt loading (with DOCA-salt) had no effect on ACE2 mRNA expression in normotensive SBN/y rats. Intriguingly, in SBH/y rats, salt loading and the development of hypertension were associated with a significant reduction in ACE2 mRNA expression as compared to normotensive

30   SBN/y rats (Fig. 2a). Of note is that ACE2 mRNA was also lower in SBH/y fed regular diet when compared to SBN/y controls fed a similar diet. This latter

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finding is consistent with the 10-20 mmHg difference in blood pressure observed between SBH/y and SBN/y rats fed normal diet.

To measure ACE2 protein levels, an ACE2 (aa206-aa225 of mouse ACE2) specific rabbit antiserum was generated, which cross reacts with both rat and
5    human ACE2 (not shown). In line with the decreased ACE2 mRNA expression, ACE2 protein expression was markedly reduced in SBH/y animals that were fed a normal diet (Fig. 2b). Increase in blood pressure of SBH/y rats following a 4-week diet of DOCA-salt correlated with further decreased ACE2 protein expression (Fig. 2b). Salt loading did not trigger
10   increased blood pressure nor did it alter ACE2 expression in salt-resistant SBN/y control rats (Fig. 2b). ACE2 protein levels were also significantly decreased in the kidneys of spontaneously hypertensive SHRSP and SHR animals as compared to their WKY controls (Fig. 2b). Moreover, the levels of ACE2 mRNA were markedly reduced in hypertensive SHRSP and SHR rats
15   (not shown). Cloning and sequencing of the coding region of ACE2 in the hypertensive rat strains did not reveal any sequence changes, indicating that reduced ACE2 expression likely results from polymorphisms that control ACE2 gene expression. The map position and reduced expression of ACE2 in three different rat strains indicate that ace2 is a strong candidate gene for this
20   hypertensive QTL on the X-chromosome. Moreover, reduced ACE2 expression in all three hypertensive rat strains suggested that this enzyme functions as a negative regulator.

## Cloning of mouse ACE2 and generation of ACE2 knock-out mice

To validate the candidacy of ACE2 as a QTL and to test whether ACE2 has inde
25   an essential role in the cardiovascular physiology and the pathogenesis cardiovascular diseases, the mouse ACE2 gene was cloned (Fig. 1a). Similar to ı and human ACE2, murine ACE2 also maps to the X-chromosome (not showı contains a single ACE-domain (Fig. 1b), and is predominantly expressed in tl kidneys and heart (Fig. 1c). Interestingly, two isoforms for ACE2 in mouse we
30   observed in all positive tissues. Overexpression of murine ACE2 in COS ce showed that ACE2 cleaves AngI into Ang1-9 (not shown) indicating that murıı

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ACE2 has the same biochemical specificity as human ACE2. To determine the vivo role of ACE2, the ace2 gene in mouse was disrupted replacing exon 9 wit neomycin resistance gene effectively deleting the zinc binding catalytic domain (: Methods and Fig. 3a). Two ES-cell lines mutated at the ace2 locus were used

5    generate chimeric mice, which were backcrossed to C57BL/6 to obtain germ transmission. Both mouse lines displayed identical phenotypes. Transmission of ace2 mutation was confirmed by Southern blot analysis (Fig. 3b). The null mutai of ace2 was verified by the absence of ace2 mRNA transcripts and proteir Northern (not shown) and Western blot analyses (Fig. 3c). ACE mRNA expressioi

10   the kidneys and hearts was not altered in ace2 mutant mice (Fig. 3d).

Since the ACE2 gene maps to the X-chromosome, all male offspring were either null mutants ($ace2^{-/y}$) or wild type for ACE2 ($ace2^{+/y}$) whereas females were either wildtype ($ace2^{+/+}$), heterozygous ($ace2^{+/-}$), or homozygous ($ace2^{-/-}$) for the ace2 mutation. It should be noted that in all experiments described

15   below, $ace2^{+/-}$ females behaved similar to $ace2^{+/+}$ females and $ace2^{+/y}$ males indicating that there is no apparent effect of ace2 heterozygosity. ACE2 null mice were born at the expected Mendelian frequency, appeared healthy, and did not display any gross detectable alterations in all organs analyzed. Moreover, in contrast to $ace^{-/-}$ male mice that display significantly reduced

20   fertility, both male and female ace2 null mice are fertile.

**Blood pressure in ace2 mutant mice**

It has been previously shown that ace2 mutant mice display reduced blood pressure and kidney pathology. Therefore, it was first tested whether loss of ACE2 expression affects blood pressure homeostasis and/or kidney

25   development or function. Intriguingly, loss of ACE2 did not result in alteration of blood pressure in 3-month old $ace2^{-/y}$ male (Fig. 4a) or $ace2^{-/-}$ female mice (not shown) as compared to their control littermates. Since it was possible that ACE could compensate for the loss of ACE2, we treated ace2-deficient mice with captopril which blocks ACE but not ACE2 function. However, in vivo

30   inhibition of ACE with captopril reduced the blood pressure of $ace^{-/y}$ male mice to a similar extent as was observed in captopril treated wild-type littermates

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(Fig. 4a). Thus, even in a scenario of ACE inhibition, loss of ACE2 has no apparent direct effect on blood pressure homeostasis in this defined mouse background. Based on our RAS QTL data, we backcross our mutant mice to other mouse backgrounds to show the role of ACE2 in blood pressure control
5    similar to genetic backgrounds in humans.

## Impaired kidney function in ace2 mutant mice

Since ACE2 is highly expressed in the kidneys, we examined kidney
10   morphology and function. All 3 month old and 6 month old $ace2^{-/y}$ male and $ace2^{-/-}$ female mice displayed normal kidney morphology and no apparent changes in any kidney ultrastructures (Fig. 4b). Normal cellularity and kidney structures of the ductal system and glomeruli were also confirmed using serial section morphometry .

15   Kidneys from male ace2 deficient mice and age-matched littermate control mice were examined using light (PAS-staining) and electron microscopy (TEM) at 3 and 12 months of age. The severity of sclerosis for each glomerulus was graded from 0 to 4+ in a blinded manner as follows: 0 represents no lesion, 1+ sclerosis of <25% of the glomerulus, while 2+, 3+,
20   and 4+ represent sclerosis of 25 to 50%, 50 to 75%, and> 75% of the glomerulus, respectively. At 3 months of age, there was no evidence of pathological changes in the kidneys from the ace2 deficient mice. However at 12 months of age, light microscopy revealed increased glomerular sclerosis/injury: $1.45\pm0.2$ vs $0.25\pm0.06$; n=6; p<0.01. Electron microscopy
25   showed increased deposition of collagen fibrils in the renal mesangium and a thickened basement membrane. The chronic exposure to elevated AngII levels leads to hypoxia and oxidative stress in the kidneys from the ace2

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deficient mice. Western blot analysis revealed increased expression of hypoxia inducible factor-1alpha (HIF1-α) and vascular endothelial growth factor (VEGF) in the kidneys from the *ace2* deficient mice. The measurement of lipid peroxidation products showed increased degree of oxidative stress in the *ace2* deficient mice at 6 months of age: hexanal ($1001 \pm 161$ vs $115 \pm 13$ nmol/g; n=5; p<0.01) and malondialdehyde ($48 \pm 6.4$ vs $24.3 \pm 2.8$ nmol/g; n=5; p<0.01).

These results show that the loss of ACE2 leads to enhanced angiotensin II signaling in a tissue-specific manner which ultimately mediates detrimental effects in the kidneys of the *ace2* deficient mice. Decreased ACE2 expression plays an important pathological role in renal disease.

## Loss of ACE2 results in a severe defect in heart function

Pharmacological inhibition of ACE or AngII receptors suggested a role for the RAS in the regulation of heart function and cardiac hypertrophy. However, neither *ace* nor *angiotensinogen* null mice develop any overt heart disease. Since ACE2 is highly expressed in the vasculature of the heart, hearts of *ace2*-deficient mice were analyzed. Hearts of *ace2* mutant mice display a slight wall thinning of the left ventricle and increased chamber dimensions (Fig. 5a). Thinning of the anterior left ventricular wall (AW) and increase in the left ventricle end diastolic dimension (LVEDD) in *ace2*-deficient hearts can be also seen by echocardiography (Table 1). These structural changes are primarily observed in 6 month old male mice. However, heart body weight ratios were comparable between age matched 3 month old (not shown) and 6 month old *ace2*$^{-/y}$ and *ace2*$^{+/y}$ mice (Fig. 5a,b). Echocardiography also showed that the left ventricle mass (LVM) and LVM/body weight ratios were normal (Table 1). Structural and biochemical changes characteristic of dilated cardiomyopathy were not observed as there was no indication of interstitial cardiac fibrosis (Fig. 5c,d) nor prototypical changes in ANF, BNP, a-MHC, b-

MHC, and skeletal muscle actin gene expression (not shown). In addition, individual cardiomyocytes of ACE2 null mice exhibited no evidence of hypertrophy and we did not observe any evidence of altered cardiomyocyte apoptosis in ACE2 null mice as detected by TUNEL staining (not shown).

5    Thus, despite mild dilation of hearts in 6 month old ACE2 null mice, there was no evidence of cardiac hypertrophy or dilated cardiomyopathy.

Interestingly, assessment of cardiac function by echocardiography revealed that all $ace2^{-/y}$ male and $ace2^{-/-}$ female mice exhibit severe contractile heart failure as determined by decreased fractional shortening (FS), and decreased

10   velocity of circumferential fiber shortening (Table 1 and Fig. 6a,b). The decrease in function was found to be more severe in 6 month old male and female mice as compared to age matched 3 month old mice, suggesting a progression in the phenotype (Table 1). Consistent with the decreased cardiac contractility, 6 month old $ace2^{-/y}$ mice exhibited reduced blood

15   pressure (Fig. 6c), a feature not found in age matched $ace2^{-/-}$ females and 3 month old males, suggesting that the reduction in blood pressure may be the result of severe cardiac dysfunction and not a direct effect of loss of ACE2 on systemic blood pressure. These surprising results show that ACE2 is a critical negative regulator of heart contractility.

20   To confirm the echocardigraphic defects in cardiac function, invasive hemodynamic measurements were performed in $ace2$ null mice. Importantly, invasive hemodynamic measurements showed that both dP/dT-max and dP/dT-min were markedly reduced in the $ace2$ mutant mice (Table 2), indicating severe impairment of contractile heart function. Loss of ACE2 also

25   resulted in a significant decrease in aortic and ventricular pressures consistent with the observed reductions in cardiac contractility (Table 2). Remarkably, the data establish that the defects in cardiac contractility of $ace2$ mutant mice occur in the absence of any overt cardiac hypertrophy and can be genetically uncoupled from alterations in blood pressure.

30   **Up-regulation of hypoxia-inducible genes in $ace2$ null mice**

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The severe contractile dysfunction and mild dilation in the absence of hypertrophy or cardiac fibrosis in *ace2* null mice resembles cardiac stunning/hibernation in humans and animal models. Cardiac stunning and hibernation are adaptive responses to chronic hypoxia such as coronary
5      artery disease or following bypass surgery. Since ACE2 is highly expressed in vascular endothelial cells but contractility is controlled by cardiomyocytes, it was speculated that loss of ACE2 could result in cardiac hypoxia. Therefore changes in the expression levels of hypoxia-inducible genes such as BNIP3[25] and PAI-1 were analyzed by Northern blotting. In the hearts of all *ace2* null
10     mice analyzed, mRNA expression of BNIP3 and PAI-1 were markedly up-regulated as compared to their *wild-type* littermates (Fig. 7a). Thus, loss of ACE2 results in the induction of a hypoxia-regulated gene expression profile.

**Increased Angiotensin II levels in tissues of *ace2* null mice**

Since ACE2 functions as a carboxypeptidase, cleaving a single residue from
15     AngI, to generate Ang1-9, and a single residue form AngII to generate Ang1-7, it was hypothesized that ACE2 may function as a negative regulator of the RAS by competing with ACE for the substrate AngI and/or cleaving and inactivating AngII. If correct, loss of ACE2 should increase AngII levels *in vivo*. Using radioimmunoassays, AngII levels were indeed found to be significantly
20     increased in the kidneys and hearts of *ace2* mutant mice (Fig. 7b). In addition, an increase in AngI was also observed (Fig. 7b) consistent with AngI being a substrate of ACE2 action *in vivo*. No differences in ACE mRNA levels were found in the hearts and kidneys of *ace2* mutant mice compared to controls indicating that the increased AngII tissue levels were not due to increased
25     ACE expression (Fig. 3d). These data show that ACE2 functions as a negative regulator of the RAS and controlling endogenous levels of AngII.

**Ablation of ACE expression in *ace2*-deficient mice rescues heart failure**

If the phenotype in the hearts of *ace2* mutant mice was due to the increase in AngII levels, then genetic ablation of ACE in combination with disruption of
30     ACE2 may serve to reduce AngII levels and rescue the phenotype observed

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in the ACE2 mutant mice. To test this notion, *ace/ace2* double mutant mice were generated. These double mutant mice were born at the expected Mendelian ratio and appear healthy. Blood pressure (Fig. 8a) and kidney defects (not shown) in the *ace-ace2* double null mice were similar to that of

5      *ace* single mutant mice. Fertility of the *ace-ace2* double mutant mice was not addressed. Thus, loss of both ACE and ACE2 does not cause any apparent disease in addition to that seen in *ace* single mutant mice.

Since the heart function of ACE knockout mice has not been previously reported the heart parameters in these mice were first analyzed. In *ace$^{-/-}$*

. 10    mice, hearts are histologically normal (not shown) and no defect in heart function could be detected at 6 months of age (Fig. 8b,c). Importantly, ablation of ACE expression on an *ace2* mutant background completely abolished the heart failure phenotype of *ace2* single knock-out mice (Fig. 8b,c). Moreover, using echocardiography, all heart functions of 6 month old, age matched *ace-*

15     *ace2* double mutant mice were comparable to that of their *ace* single mutant and *wild type* littermates (Table 1). Restoration of heart functions occurred in both male and female *ace-ace2* double mutant mice. These genetic data show that ACE expression is required and necessary to trigger contractile heart failure in the absence of ACE2.   Importantly, there was also no

20     difference in blood pressure between *ace* and *ace/ace2* knockout mice (Fig. 8a), further implying that the reduced blood pressure in older male *ace2* mice is due to the dramatic decrease in heart function.

**ACE2 negatively controls lung injury**

Adult respiratory distress syndrome (ARDS) is a serious form of acute lung

25     injury and has mortality rates of 40-70% even when intensive care is available. Trauma, severe sepsis (systemic infection), diffuse pneumonia and shock are the most serious causes of ARDS, and among them acid-induced

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lung injury is one of the most common causes. Potential mechanisms causing acid aspiration-associated lung injury include HCl-induced damage to the alveolar-capillary membrane, and polymorphonuclear neutrophil (PMN) adhesion, activation and sequestration, which results in pulmonary edema
5   and the deterioration of gas exchange. Treatment for ARDS consists of mechanical ventilation and continuing treatment of the precipitating illness or injury. The supportive treatment using novel drugs protecting lung from further alveolar-capillary membrane damage would preserve the ARDS patients' lung function in order to treat the precipitating illness or injury, leading to a
10  decrease in the mortality rate of ARDS.

Since ACE2 is expressed in lungs, we assessed whether loss of ACE2 has any role in acute lung injuries. Figure 9 shows the changes in lung elastance (EL) in HCl-treated and control mice over a 3 hour period. Following HCl administration, wild type mice survived more than 4 hours, whereas ACE2
15  knockout mice died within 2-3 hours. Reflecting the difference of survival, ACE2 knockout mice showed a significantly more severe response in lung elastance than wild type mice. Thus, ACE2 plays a significant role in protecting lungs from acute acid-induced injury. Thus, enhancing ACE2 function and/or expression is a novel and unanticipated target for the
20  treatment for ARDS and lung disease.

**Methods**

**Cloning of mouse and rat ACE2 and chromosomal QTL mapping.** Murine ACE2 was cloned from a proprietary EST database. Using a mouse ACE2
25  probe, we then screened a rat kidney cDNA (Invitrogen) to obtain a full-length rat cDNA as determined by DNA sequencing. For chromosomal mapping, a rat *ACE2* cDNA specific probe was used to screen a rat PAC library (RPCI-31, Research Genetics), identifying two positive clones (6M6 and 125K9).

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The end sequences of these clones were determined and rat specific primers were designed (mc2L: 5'-TCAATTTACTGCTGAGGGGG-3', mc2R: 5'-GAGGGATAACCCAGTGCAAA-3') to determine the chromosomal map position of *ACE2* in rat by screening a radiation hybrid panel (RH07.5,

5   Research Genetics). SHR and control WKY rats were obtained from Harlan and maintained at the animal facilities of the Ontario Cancer Institute in accordance with institutional guidelines. Tissues from SHRSP rats were kindly provided by Dr. Detlev Ganten, Germany. Salt-resistant and salt-sensitive Sabra rats were bred and maintained at the animal facility of the Ben-Gurion

10  University Barzilai Medical Center, Israel. Doca-salt treatment was as described previously.

**Expression analysis.** Total RNA was prepared form rat kidneys using tri-reagent. 20 mg of RNA was resolved on a 0.8% formamide gel. Blotted to nylon membrane (Amersham), and probed with a partial rat ACE2 cDNA

15  clone (9-1). The β-actin probe and Multiple tissue Northern blots were purchased from Clontech. For western analysis, kidneys were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 20mM EDTA, and 1% triton-X 100) supplemented with "Complete" protease inhibitor cocktail (Roche) and 1 mM $Na_3VO_4$. 100 mg of protein was resolved by SDS-PAGE on 8% tris-glycine

20  gels. ACE2 immuno-serum was obtained from rabbits immunized with a mouse specific ACE2 peptide DYEAEGADGYNYNRNQLIED. The serum was affinity purified with the immunizing peptide using sulpho-link kit (Pierce). A commercially available β-actin antibody was used as loading control (Santa Cruz).

25  **Generation of ACE2 mutant mice.** A targeting vector (559 base pair short arm, and 8.1 kilobase long arm) was constructed using the pKO Scrambler NTKV-1907 vector (Stratagene). A portion of the *ace2* genomic DNA containing nucleotides +1069 to +1299 was replaced with the neomycin resistance cassette in the anti-sense orientation. The targeting construct was

30  electroporated into E14K ES cells, and screening for positive homologous recombinant ES clones was performed by Southern blotting of EcoRI-digested

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genomic DNA hybridized to 5' and 3' flanking probes. Two independent *ace*[-/y]
ES cell lines were injected into C57BL/6-derived blastocysts to generate
chimeric mice, which were backcrossed to C57BL/6 mice. Two ES cell lines
gave independent germline transmission. Data reported in this manuscript are
5  consistent between the two mutant mouse lines. Ablation of ACE2 expression
was confirmed by RT-PCR, Northern, and Western blot analyses. Only
littermate mice were used for all experiments. Histology of all tissues,
apoptosis assays, blood serology, and kidney morphometries were as
described[31]. Complete ACE mutant mice have been previously described[8]
10  and were obtained from Jackson Laboratories. Mice were maintained at the
animal facilities of the Ontario Cancer Institute in accordance with institutional
guidelines.

**Heart morphometry, echocardiography, hemodynamics and blood
pressure measurements.** For heart morphometry, hearts were perfused with
15  10% buffered formalin at 60 mmHg and subsequently embedded in paraffin.
Myocardial interstitial fibrosis was determined by quantitative morphometry
using the color-subtractive computer assisted image analysis using Image
Processing Tool Kit version 2.5 coupled with Photoshop 6.0 software. Picro-
Sirius red stained sections were used to calculated interstitial fibrosis as the
20  ratio of the areas with positive PSR staining compared to the entire visual
field. Echocardiographic assessments were performed as described[32] using
*wild-type* and *mutant* littermates. Mice were anesthetized with
isoflourane/oxygen and examined by transthoracic echocardiography using a
Acuson® Sequoia C256 equipped with a 15MHz linear transducer. FS was
25  calculated as: FS = [(EDD − ESD)/EDD]*100. Vcfc was calculated as
FS/ejection time corrected for heart rate. Hemodynamics measurements were
performed as described. Briefly, mice were anesthetized , and the right carotid
artery was isolated and cannulated with a 1.4 French Millar catheter (Millar
Inc., Houston) connected to an amplifier (TCP-500, Millar Inc.). After insertion
30  of the catheter into the carotid artery, the catheter was advanced into the
aorta and then into the left ventricle to record the aortic and ventricular
pressures. The parameters measured and analyzed were heart rate, aortic

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pressure, left ventricular (LV) systolic pressure, LV diastolic pressure, and the maximum and minimum first derivatives of the LV pressure (+dP/dtmax and dP/dtmax, respectively). Tail-cuff blood pressure measurement were taken using a Visitech BP-2000 Blood Pressure Analysis System manufactured by
5   Visitech Systems (Apex, NC).  For captopril treatment, drinking water was supplemented with 400mg/L captopril (Sigma) for two weeks prior to blood pressure measurement.

**Tissue angiotensin peptide levels.** Hearts and kidneys were homogenized on ice in 80% ethanol /0.1 N HCl containing the peptidase inhibitors described
10  above including phenylmethylsulfonyl fluoride (PMSF, 100 µM). Protein homogenates were centrifuged at 30,000 $g$ for 20 minutes, supernatants decanted, and acidified with 1% (v/v) heptafluorobutyric acid (HFBA, Pierce, Rockford, IL). The supernatant was concentrated to 5 ml on a Savant vacuum centrifuge (*Savant*, Farmingdale, NY) and concentrated extracts were applied
15  to activated Sep-Paks, washed with 0.1% HFBA, and eluted with 5 ml 80% methanol / 0.1% HFBA. Radioimmunoassay analysis of angiotensin peptide content in the extracts from heart and kidney tissues was performed. The limits of detection for the Ang II and Ang I RIAs were 0.5 fmol/tube and Ang I 5 fmol/tube, respectively.

20  **Acute lung injury model.** ACE2 knockout mice and their littermates (8-12 weeks old) were used in this study. One minute before aspiration challenge, 2 deep inhalations (3 times tidal volume) were delivered to standardize volume history and measurements were made as baseline. Anesthetized and mechanically ventilated mice received intratracheal injection of 2 mL/kg HCl
25  (pH = 1.5), followed by a bolus of air. In the control group, mice received saline injection or no injection. In all groups, measurements were made at 30-minute intervals for 3 hours. To assess lung injury physiologically, lung elastance (EL; a reciprocal of lung compliance) was evaluated by measuring

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the tracheal peek pressure, flow, and volume. EL was calculated by dividing tracheal peek pressure with volume. Changes in EL reflect lung parenchymal alterations and stiffening of the lungs.

5      The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made without departing from the spirit and scope thereof.   For example, where the application refers to proteins, it is clear that peptides and polypeptides may

10     often be used.  Likewise, where a gene is described in the application, it is clear that nucleic acid molecules or gene fragments may often be used.

All publications (including Genbank entries), patents and patent applications are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and

15     individually indicated to be incorporated by reference in its entirety.

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Table 1. Heart functions of ace2 null mice

| | 3 month - males | | 6 month - males | | 6 month - females | |
|---|---|---|---|---|---|---|
| | ace2+/y | ace2-/y | ace2+/y | ace2-/y | ace2+/- | ace2-/- |
| | n=7 | n=7 | n=8 | n=8 | n=3 | n=3 |
| Heart Rate, bpm | 469 ±12 | 466 ±18 | 495 ±15 | 482 ±12 | 460 ±6 | 452 ±14 |
| AW, mm | 0.65 ±0.02 | 0.62 ±0.01 | 0.66 ±0.01 | 0.59 ±0.02* | 0.65 ±0.02 | 0.57 ±0.04 |
| LVEDD, mm | 4.09 ±0.04 | 4.25 ±0.10 | 4.12 ±0.10 | 4.49 ±0.12* | 3.71 ±0.10 | 4.11 ±0.07* |
| LVESD, mm | 2.11 ±0.04 | 2.69 ±0.09** | 2.13 ±0.06 | 3.29 ±0.14** | 1.74 ±0.07 | 2.68 ±0.11** |
| % FS | 48.42 ±1.14 | 36.73 ±0.89** | 48.12 ±0.84 | 26.76 ±1.78** | 53.00 ±1.68 | 34.85 ±1.76** |
| PAV, M/s | 0.992 ±0.029 | 0.922 ±0.033 | 0.902 ±0.044 | 0.802 ±0.037 | 0.875 ±0.048 | 0.809 ±0.044 |
| Vcfc, circ/s | 9.49 ±0.48 | 7.28 ±0.45* | 9.46 ±0.26 | 4.93 ±0.33** | 10.34 ±0.81 | 6.16 ±0.31** |
| LVM, mg | 95.69 ±1.88 | 95.50 ±2.28 | 102.41 ±4.00 | 100.49 ±3.39 | 92.27 ±2.02 | 89.60 ±1.54 |
| LVM/BW, mg/g | 3.32 ±0.08 | 3.53 ±0.18 | 3.19 ±0.10 | 3.32 ±0.15 | 3.29 ±0.37 | 3.30 ±0.09 |

*$p < 0.05$ ace2-/y vs ace2+/y or ace2-/- vs ace2+/-

**$p < 0.01$ ace2-/y vs ace2+/y or ace2-/- vs ace2+/-.

Bpm = heart beats per minute; AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension; LVESD = left ventricle end systolic dimension; %FS = percent fractional shortening; PAV = peak aortic outflow velocity; Vcfc = Velocity of circumferential fiber shortening; LVM = calculated left ventricular mass; BW = body weight.

**Table 2. Invasive hemodynamic parameters of 6 months old ace2$^{-/y}$ mice**

| | ace2$^{+/y}$ | ace2$^{-/y}$ |
|---|---|---|
| | N = 8 | N = 8 |
| Heart rate, bpm | 303 ±16 | 298 ±8 |
| SBP, mmHg | 111.5 ±2.4 | 91.6 ±3.0 |
| DBP, mmHg | 70.5 ±3.0 | 50.3 ±2.4** |
| MBP, mmHg | 84.2 ±2.8 | 64.0 ±2.6** |
| LVSBP, mmHg | 107.4 ±4.5 | 87.5 ±2.3** |
| LVEDBP, mmHg | 5.5 ±0.8 | 5.3 ±0.7 |
| dP/dT max | 5579 ±422 | 3034 ±124** |
| dP/dT min | -5055 ±257 | -2929 ±271** |

**p < 0.01 ace2$^{-/y}$ vs ace2$^{+/y}$

SBP = systolic blood pressure; DBP = diastolic blood pressure; MBP = mean arterial blood pressure; LVSBP = left ventricle systolic blood pressure; LVEDBP = left ventricle end diastolic blood pressure; dP/dT max = maximum 1st derivative of the change in left ventricular pressure/time; dP/dT min = minimum 1st derivative of the change in left ventricular pressure/time

**Table 3. Heart functions of 6 months old ace and ace/ace2 null mice**

|              | ace^{-/-}        | ace^{-/-} ace2[-/y]  |
|--------------|-----------------|---------------------|
|              | n=8             | n=8                 |
| Heart Rate, bpm | 507 ±17      | 491 ±10             |
| AW, mm       | 0.63 ±0.01      | 0.65 ±0.02          |
| LVEDD, mm    | 3.86 ±0.04      | 3.79 ±0.07          |
| LVESD, mm    | 2.11 ±0.04      | 2.13 ±0.07          |
| % FS         | 45.34 ±1.11     | 43.95 ±1.24         |
| PAV, M/s     | 0.995 ±0.064    | 0.931 ±0.040        |
| Vcfc, circ/s | 8.94 ±0.25      | 8.40 ±0.27          |

AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension; LVESD = left ventricle end systolic dimension; %FS = percent fractional shortening; PAV = peak aortic outflow velocity; Vcfc = Velocity of circumferential fiber shortening.